Nucleic acid immunization: a prophylactic gene therapy?

Klaus Cichutek

Nucleic acid (NA) vaccines may offer the safety of subunit or inactivated vaccines and, at the same time, provide the advantages of live recombinant vaccines, such as induction of a protective cellular immune response. In Germany, the so-called 'Gene Law' regulates the genetic modification of organisms such as prokaryotic or eukaryotic cells for the construction of recombinant NAs intended for use as NA vaccines. Neither NAs nor human beings treated with NAs are subject to Gene Law regulations but preclinical laboratory experiments are regulated by the Gene Law. Gene therapy, as defined in a recent draft of a European guideline for the production of gene therapeutics, includes the genetic modification of human somatic cells via transfer of NAs and thus includes NA vaccines. The guideline provides recommendations for the production of NA vaccines for human use and for preclinical safety testing. NA vaccines are products derived by biotechnological processes, as defined in part A of the annex of Council Regulation (EEC) No. 2309/93 of 22 July 1993. Applications for marketing authorization in Member States of the European Union will thus be reviewed by the European Agency for the Evaluation of Medicinal Products starting from 1 January 1995. Inoculation of NAs encompassing a full-length but int/nef*-defective simian immunodeficiency provirus allowing limited replication of viruses released is being investigated at the Paul-Ehrlich-Institute as a model for a NA vaccine against AIDS. The system may offer a promising way towards a vaccine and will also be used to study safety requirements for future human use of NA vaccines.*

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GENE THERAPY AND NUCLEIC ACID IMMUNIZATION: A COMPARISON

Therapies that are aimed at, or at least take into account, the temporal genetic modification of human somatic cells have been used for some time. Novel genetic therapies are directly aimed at modifying the pattern of expression of specific genes within a cell. Some of them, e.g. anti-sense nucleic acid (NA) treatment, are mainly reversible as the previous pattern of gene expression is probably restored when the treatment is discontinued. In contrast, human somatic gene therapy is defined as the transfer of genes or the correction of existing genes, with the goal of achieving genetic modification in human somatic cells for a prolonged period of time. As well as gene addition by transfer of NAs encompassing one or more complete genes, the transfer of NAs aimed at correcting the structure or function of genes by homologous recombination will be possible[1,2]. NA transfer is performed either *in vivo* (e.g. by administration of replication-incompetent adenoviruses transferring the cystic fibrosis transmembrane conductance regulator (CFTR) gene into airway epithelium through aerosols) or *ex vivo* (e.g. by retroviral transduction of peripheral blood cells with adenosine deaminase gene expression vector and subsequent intravenous (i.v.) infusion of the patient's corrected cells into the blood). Both procedures illustrate that the techniques that are currently available lead to extrachromosomal or chromosomal persistence of the transferred gene within somatic cells and thus to gene addition. Gene transfer vectors used include defective viruses that are replication-incompetent and apathogenic by deletion of defined viral genomic sequences. They deliver expression vectors, which encompass genes intended for expression. Expression vectors also contain sequences necessary for packaging and completion of a single round of viral replication after infection, a process termed transduction of cells. Carrier-free NAs or NAs associated with physicochemical carriers may also be used for gene transfer, resulting in transfection of the targeted cells. This will also result in either extrachromosomal persistence of the expression constructs or chromosomal integration, depending on the technique used. For example, receptor-mediated uptake by transferrinfection[3] may lead to extrachromosomal persistence of large amounts of DNA and a probably low frequency of DNA integration. The expression of proteins from the expression vectors is aimed at correction of the defect and may or may not include an immune reaction

Department of Biotechnology, Paul-Ehrlich-Institute, Paul-Ehrlich-Strasse 51–59, D-63225 Langen, Germany

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of the host towards the gene product. During tumour vaccination, induction of a vigorous immune response against the transduced human somatic cells is actually the goal of the procedure.

Immunization by transfer into human somatic cells of NAs not associated with physicochemical complexes or viral vectors that comprise complete genes is therefore only a specific form of gene transfer/therapy[4–14]. Carrier-free NAs, NAs bound to gold beads or mixed with so-called facilitators are incorporated into cells after intramuscular (i.m.) or i.v. inoculation or via other routes. Subsequently, a persistent expression of the respective gene constructs is observed for a prolonged period of time in the host, particularly in i.m. inoculations. The recombinant NA will comprise one or more complete genes in addition to vector sequences used for production, e.g. in bacterial hosts. The NAs are taken up by cells residing in the muscle and are expressed from extrachromsomal DNA, although integration of the constructs has not been completely excluded. The purpose of gene transfer by uncomplexed recombinant NAs is the immunization of the host. It thus comprises prophylactic immunization, as in vaccination against infectious diseases, or therapeutic immunization, as possibly necessary for the treatment of AIDS. Therefore, recombinant NA immunization displays hallmarks of prophylactic immunization by live recombinant viruses as well as therapeutic immunization, as planned for use during gene therapy of HIV-infected patients.

The regulatory issues of gene therapy are currently being discussed in various Directorates of the European Union (EU) as well as in various political and scientific institutions of Member States like Germany. The first two gene therapies in Germany have recently been carried out in Berlin and Freiburg. They involved transfer of cytokine genes into malignant tumour cells for tumour vaccination. A few European companies have started to develop, in association with scientists in the field of gene therapy vector development, products that will be used for the standard treatment of patients. NA immunization has been recognized as an interesting field from the perspective of development of vaccines that are easy to use and handle. The emerging picture of the future regulatory procedures for clinical testing and marketing of NA vaccines in the EU and Germany as described here is based on discussions during the 'EC Workshop on Human Somatic Gene Theapy' held by the Directorate General (DG) XII (for Science, Research and Technology) at the NIBSC in London, discussions of the drafting group of the '*Ad-hoc* working party on biotechnology/pharmacy' by DG III (Industry), which recently released for comments draft III of the European Note for Guidance termed 'Gene Therapy Products—Quality, Safety and Efficacy Aspects in the Production of Vectors and Genetically Modified Somatic Cells', and discussions during the meeting 'Non-Target Effects of Live Recombinant Vaccines' at the Paul-Ehrlich-Institute in Langen, Germany, held in 1993 under the auspices of the OECD. In Germany, an *ad hoc* working group 'Gene Therapy', organized by the Federal Ministry of Health, formally discusses scientific, ethical and legal aspects of gene therapy, involving the various federal German states (Länder). In summary, scientific and regulatory approaches for the introduction of NA immunization and other gene therapies as standard vaccination procedures or therapies are currently being discussed and will lead to identification of the necessary regulatory steps to be taken to allow introduction and legal use of the procedures in the EC. However, the procedure for applying for marketing authorization of future NA vaccines has already been defined and will be outlined below.

PRECLINICAL AND CLINICAL SAFETY TESTING UNDER THE REGULATIONS OF THE GERMAN GENE LAW

General recommendations for clinical safety testing are given in the Guideline 'Recommended Basis for the Conduct of Clinical Trials of Medicinal Products in the EC'. Preclinical laboratory and animal experiments involving genetically modified organisms (GMOs) such as prokaryotic or eukaryotic cells, viruses, parasites and animals modified by methods of recombinant DNA technology have to be performed under containment according to EU Directives 90/219/EEC of 2 April 1993 and 90/220/EEC of 23 April 1993. These directives are translated into related laws specific for each Member State of the European Union. The German law for the contained use of GMOs, abbreviated as the 'Gene Law', was introduced in July 1990 and revised to its current version in December 1993. It specifically states that the genetic modification of human beings is not subject to the regulations of the Gene Law. The Gene Law also regulates obtaining consent from the national competent authorities for the deliberate release of GMOs for research or placing on the market of products containing or consisting of GMOs according to directive 90/220/EEC. GMOs for which marketing authorization has been granted are no longer subject to regulation by the law. Thus, work with licensed, live recombinant vaccines or humans vaccinated with live recombinant vaccines is not subject to gene regulations.

Obviously, NAs not packaged into replicating organisms are not considered as GMOs according to the Gene Law. As mentioned earlier, human beings inoculated with NAs alone or in the presence of non-viral carriers are not GMOs according to the Gene Law. However, medical treatment with GMOs, e.g. during somatic cell therapy with genetically modified cells, has so far been performed within a special gene laboratory. Permission for the use of such a facility and for the specific genetic experiment has to be granted by authorities of the German states (Länder), which will prove impractical if standard gene therapy procedures are to be performed by medical doctors who do not themselves perform the laboratory experiments needed to prepare the GMOs used for the patients' treatment. This, however, is of no concern for NA vaccination as NAs and NA-vaccinated humans will not be considered as GMOs.

CONCERTED PROCEDURE FOR APPLICATION FOR MARKETING AUTHORIZATION OF NA VACCINES

The EU procedures for obtaining marketing authorization for gene therapeutics have been defined. Medicinal products as defined in part A of the annex of Council Regulation (EEC) No. 2309/93 are '... developed by means of one of the following biotechnological processes: recombinant DNA technology, controlled expression of genes coding for biologically active proteins in

prokaryotes and eukaryotes including transformed mammalian cells ...'. This definition seemingly encompasses future products for human somatic gene transfer, such as viral or non-viral gene transfer vectors and carrier-free NAs, irrespective of their use as therapeutics or vaccines. Starting from 1 January 1995, applications for marketing authorization of NA vaccines will thus be reviewed by the European Agency for the Evaluation of Medicinal Products. The application procedure is designed so as to fulfil the criteria of the 'one door/one key' principle, allowing short review times and granting of marketing authorization in all Member States of the EC after submission of a single application. According to this procedure, authorization is granted by the Commission within 240 days. The notification of the marketing authorization is published in the 'Official Journal of the European Communities'. The application format and procedures for application are outlined in 'The Rules Governing Medicinal Products in the European Community', e.g. in Volume IIA 'Notice to Applicants for Marketing Authorisation for Medicinal Products for Human Use in the European Community'. The outline of the legal basis for the procedures is given in Volume I ('Rules Governing Medicinal Products in the European Community') (Office for Official Publication for the European Communities, 2 Rue Mercier, L-2985 Luxembourg). Procedures are based, for example, on Council Regulation (EEC) No. 2309/93 of 22 July 1993 and Council Directive 93/39/EEC of 14 June 1993.

Basically, the procedure involves the submission of an application dossier to the European Agency for the Evaluation of Medicinal Products, which will be located in London. The application should be written on the basis of Guidelines and Notifications to the applicants, as given in the published rules cited above. It is anticipated that the review process will be started by selection of a rapporteur and possibly a co-rapporteur from the members of the Committee for the Evaluation of Proprietary Medicinal Products (CPMP) or members of the Committee for the Evaluation of Veterinary Medicinal Products (CVMP). The rapporteur and co-rapporteur will examine the application for safety, efficacy, quality and for possible environmental risks and will therefore most likely select expert assessors with known scientific and regulatory backgrounds for the primary review process. Within 210 days, an assessment report will be prepared by the rapporteur that combines all reviewed aspects of the product and forms the basis for the decision of the Community to grant of marketing authorization for the respective medicinal product. The rapporteurs will also prepare a draft summary of the product characteristics, give details of any possible restrictions and draft the text for the labelling and packaging leaflet. Thus they will provide the basis for the decision of the Commission to grant marketing authorization for a specific medicinal product.

THE GUIDELINE FOR GENE THERAPY PRODUCTS APPLIES TO NA VACCINES

Gene therapy, as defined in a recently completed third draft of a European Note for Guidance for the production of gene therapeutics ('Gene Therapy Products – Quality, Safety and Efficacy Aspects in the Production of Vectors and Genetically Modified Somatic Cells'), includes the genetic modification of human somatic cells via transfer of NAs that either encompass complete functional genes or that are intended to modify the structure or function of endogenous genes by recombination. This definition seemingly includes NA vaccines. Immunization by application of NA vaccines may serve prophylactic or therapeutic purposes.

Quality, efficacy and safety considerations apply to all biological products manufactured by recombinant DNA technology, including those intended for NA immunization and other gene therapies. Products intended for NA vaccination will be controlled by a flexible approach and any recommendations will be adopted for future developments. The intended clinical use of a product will form the basis for any case-by-case evaluation.

Points to consider in manufacture

Establishments where NA vaccines will be manufactured have to meet the requirements of EC Directive 91/356/EEC of 13 June 1991 on GMP and 90/219/EEC of 23 April 1990 on contained use of genetically modified micro-organisms. All reagents used in production should comply with relevant EU and ICH recommendations (Notes for Guidance, e.g. 'Stability'). Agents known to provoke sensitivity in individuals, such as certain antibiotics, should be avoided during manufacture. The final product should be characterized and the principles of 'in-process' control should be used to maintain quality standards.

Special attention should be given to the amplification system used for production of the NA to be inoculated. The replication system should provide high fidelity or should be controlled for the resulting quality and homogeneity of the product. The biological material containing the cloned gene should be controlled thoroughly before use. This should include all components of the final product, not only the expression construct. After gene transfer, the gene should not produce pathological effects as such. Adventitious agents and NAs possibly resulting from production should not be present. Specifically, adventitious NAs and sequences that allow recombination with oncogenes or tumour-suppressor genes should be avoided. Sequences needed for amplification, e.g. in bacterial cells, should not be expressed in cells. Impurities in the final product may depend on the manufacturing and purification procedures chosen. For example, endotoxins, as typical by-products of manufacturing processes using bacterial cells, should be removed. Variability in the cultures during production may lead to variability in the consistency of the final product, and thus procedures ensuring product consistency are imperative. Scale-up of the production or purification schemes may have significant consequences for the product and thus have to be thoroughly controlled for their effects on the final product. Thus the product has to be characterized biochemically as well as genetically. All considerations will be applied on a case-by-case basis to the products under review.

Genetic considerations

The genetic origin of the expression products, including cloning and amplification of the gene(s), must be taken into consideration. Full details of the origin, identification and isolation as well as amplification of the nucleotide sequences to be transferred should be indicated. If the NA to be transferred is to be physically

coupled to other material that is necessary for its effect, this material has to be considered separately. An example would be the coupling of peptides to NAs for receptor-mediated uptake into cells. Here, peptide synthesis, characterization, the coupling process and consistency of the final product will have to be thoroughly controlled. If possible, all materials used should be of pharmaceutical quality. Where selection techniques are required to isolate cells, details of the method should be provided.

During production, details of the process, including volumes, times, harvest and storage, should be given. The 'batch' of the product should be clearly defined. Acceptable limits for the purity, consistency and yield of the product should be specified. A complete description of the methods used in purification should be provided in detail. Also, the capacity of purification methods to remove potential contaminants should be investigated.

The final product has to be thoroughly characterized. The stability should be demonstrated by a number of biological and molecular techniques. The expression construct should have the biochemical and biological characteristics desired and required for its use. The purity of the final product should be demonstrated and correlated with the defined acceptable limits of impurities. Acceptance or rejection criteria for a batch should be defined. It is evident that inclusion of contaminating micro-organisms and viruses has to be thoroughly avoided.

Preclinical safety testing

Preclinical safety testing has to be aimed at identifying any unintended and unexpected consequences of the gene transfer. General recommendations are found in the Guidelines 'Pre-clinical Biological Safety Testing of Medicinal Products Derived from Biotechnology'. An undesired genetic consequence of NA vaccination may be the insertional mutagenesis of cellular genes due to a low level of integration of the expression construct, leading to an increased risk of cancer. In addition, a high copy number of unintegrated genes may be cause for concern. Cellular changes that may be induced by the gene transfer should be thoroughly analysed. Reactivation of any viruses already present in the cells into which the expression construct is to be transferred may be problematic. Immunogenicity of the cells that express the transferred construct may induce autoimmunity against these cells and therefore should be known for their potential risk and harmful effects. The expression of other than the desired sequences from the construct should be avoided, if possible, or at least reduced to a minimum. Recombination with viruses present may lead to mobilization of the expression construct, which is to be avoided. Non-target cells may incorporate the expression construct, and horizontal spread to other humans or into the germ line cannot be accepted at any level.

Preclinical safety testing as described in the Guideline 'Pre-clinical Biological Safety Testing of Medicinal Products Derived from Biotechonology' (Vol. III, 'The Rules Governing Medicinal Products in the European Community') should be considered. Safety testing in animals is imperative whenever possible as the NAs will be directly applied *in vivo*. Acute toxicity and pharmacokinetic tests, and tests for chronic toxicity and tumorigenicity should be performed in animals when necessary.

SIVΔ*int*/Δ*nef*: A MODEL FOR A VACCINE AGAINST AIDS?

Since progress in the development of a 'conventional' vaccine against AIDS is slow, NA vaccines should be analysed for their capability to protect against HIV infection or disease. Studies at the Paul-Ehrlich-Institute have included infection of African green monkeys (AGM; *Cercopithecus aethiops*) with simian immunodeficiency virus SIV_{agm} and infection of rhesus macaques (*Macaca mulatta*) with SIV_{mac}[15–20]. In the SIV_{agm} system, whole inactivated virus, synthetic peptide immunogens and passive transfer of antibodies from naturally infected AGM to seronegative AGM resulted in no protection after i.v. challenge with homologous SIV_{agm}. Whole inactivated virus immunogen repeatedly led to 100% protection of rhesus macaques after challenge with SIV_{mac} cultivated on the same T-cell lines used for production of the immunogen. Experiments by Stott[21] indicated that protection was mainly due to a protective immune response against cell proteins. Subsequently, live attenuated SIV_{mac}-239Δnef was shown to protect animals from infection by homologous and heterologous SIV_{mac} by Desrosier and co-workers[22,23]. Thus live attenuated lentiviruses are the only promising candidates for a vaccine against AIDS at the moment. However, use of live attenuated HIV as a vaccine for humans may be difficult.

Recent studies using other live attenuated SIVs suggest that prolonged and low-level infection is needed to produce protection. We therefore established severely attenuated mutants of SIV_{mac} that only accomplish a single round of replication but induce expression of viral antigens in transfected or infected cells (*Figure 1*)[20]. Deletions were introduced into replication-competent full-length molecular clones of SIV_{mac}. All mutants express a truncated version of the *nef* gene product, which is presumed to be necessary for high-level infections *in vivo*. In addition, single deletions were introduced into the integrase domain of the *pol* gene. Viruses were generated by transfection of three different *int*/*nef*-double mutant proviruses into HeLa tat-III cells and viral antigens were shown to be expressed. The transfected cells were subsequently co-cultivated with CD4+ permissive T-cell lines using semipermeable membranes. After 2–3 days of co-cultivation, infection by SIVΔint/Δnef was demonstrated using immune peroxidase staining of infected cells. Single cells were stained using sera of SIV_{mac}-infected monkeys. Amplification of a specific region of the *pol* gene starting from DNA of the infected T cells was used to demonstrate production of viral DNA in the infected cells. After separation of extrachromosomal from chromosomal DNA, no SIV-specific DNA fragment could be amplified from the chromosomal DNA, whereas specific DNA amplicons were obtained from the extrachromosomal fraction of the cell DNA. As expected, SIVΔ*int*/Δ*nef* viruses were able to enter T cells and transcribe viral RNA into viral DNA, which was then probably incorporated into the nucleus. Expression of viral antigen was demonstrated within the first 3 days of infection. Therefore, *int*/*nef*-double mutant viruses are able to undergo a single round of infection and express antigen from extrachromosomal viral DNA. Infection is limited and abortive, as viral DNA is probably exported from the cells by unknown mechanisms.

Spread of SIVΔ*int*/Δ*nef* from transfected muscle cells

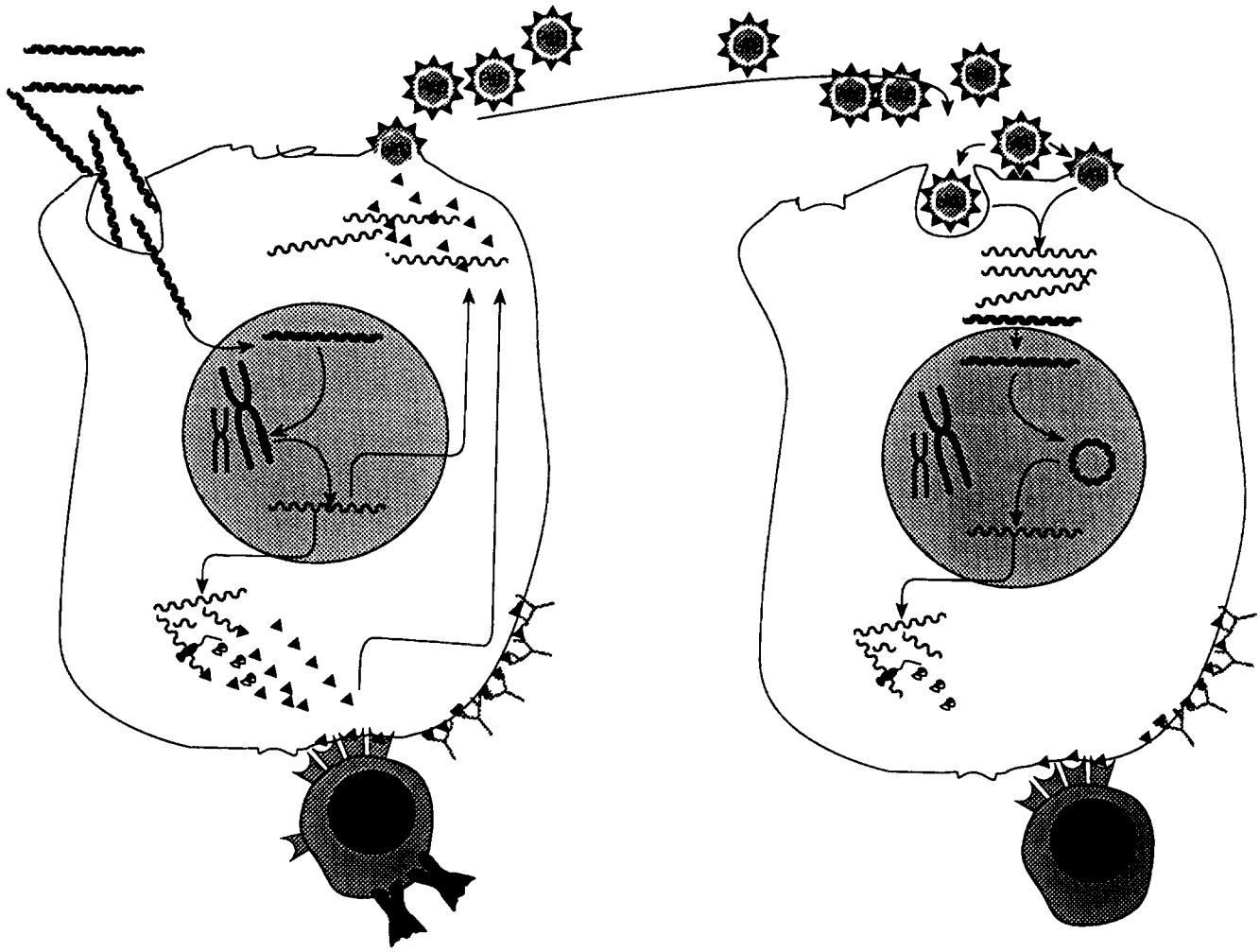

Figure 1 Limited replication by integrase-negative mutants of simian immunodeficiency virus (SIVΔ*int*) after proviral DNA transfer into human somatic cells[20]. On the left is shown a human somatic cell which may or may not integrate proviral DNA of SIVΔ*int* after DNA inoculation, e.g. into muscle. Production of virus particles was shown *in vitro* to be sufficient for the transduction (single round of replication without provirus integration) of neighbouring cells for a limited period of time due to the absence of chromosomal integration of the viral DNA. An immune response may be induced by viral antigen presentation of the primary cells (left) which take up the DNA inoculated during NA vaccination or by the secondary cells (right) transduced by viruses with limited replication capacities

to CD4+ cells could provide an efficient means to induce an immune response. Therefore, double mutant proviruses were recently injected into the muscle of Balb/c mice and expression of viral antigens was demonstrated by immune staining of transfected cells. Experiments using rhesus macaques will give us the opportunity to achieve boosting of the immune response, as monkeys are permissive for SIV replication. Immunization by inoculation of NAs encompassing a full-length but *int*/*nef*-defective SI provirus, allowing limited replication of virus particles released, may thus offer a promising way towards a vaccine against AIDS.

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